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Article

Krempfielins J–M, New Eunicellin-Based Diterpenoids from the Soft Coral *Cladiella krempfi*

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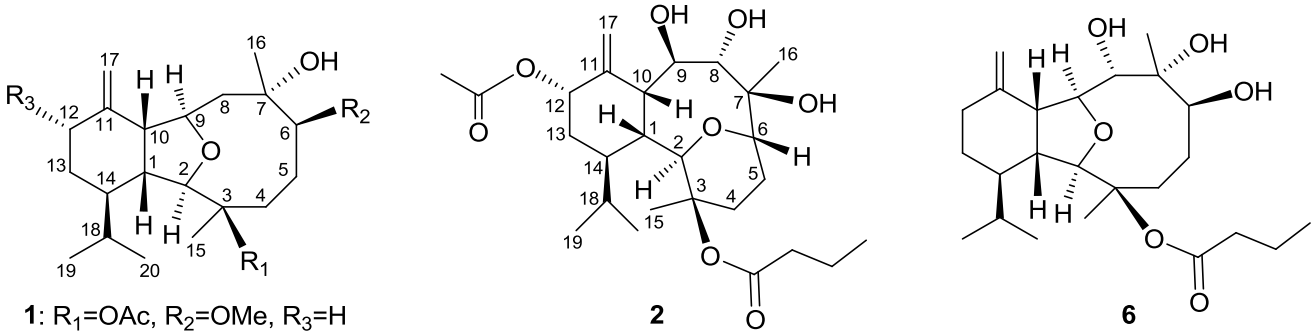
Abstract: New four eunicellin-based diterpenoids, krempfielins J–M (**1**–**4**) were isolated from the organic extract of a Taiwanese soft coral *Cladiella krempfi*. The structures of the new metabolites were elucidated on the basis of extensive spectroscopic analysis. The structure of compound **2** is rare due to the presence of the highly oxygenated pattern. Anti-inflammatory activity of **1**–**6** to inhibit the superoxide anion generation and elastase release in FMLP/CB-induced human neutrophils was also evaluated, and **2** and **4** were shown to possess the ability to inhibit the elastase release.

Keywords: *Cladiella krempfi*; eunicellin-based diterpenoid; elastase; anti-inflammatory agent

1. Introduction

Many recent studies about the discovery of versatile structures and bioactivities of eunicellin-type compounds isolated from soft corals have been reported [1–15]. The soft coral *Cladiella krempfi* has been found to generate several types of metabolites including eunicellin-type diterpenoids [16,17] and pregnane-type steroids [18–20]. Our previous study on the bioactive secondary metabolites of a Taiwanese soft coral *Cladiella krempfi* also resulted in the isolation of a series of new eunicellin-based diterpenoids, krempfielins A–I [21,22]. In this paper, we further report the discovery of four new eunicellin-based diterpenoids, krempfielins J–M (**1**–**4**), along with two known diterpenoids sclerophytin F (**5**) [23] and litophynol B (**6**) [24] (Chart 1). The ability of these compounds to inhibit the superoxide anion generation and elastase release in FMLP/CB-induced human neutrophils was also evaluated. The results showed that at 10 μM compounds **2** and **4** effectively inhibited the elastase release in FMLP/CB-induced human neutrophils.

Chart 1. Structures of metabolites **1**–**6**.



1: R_1=OAc, R_2=OMe, R_3=H
3: R_1=OAc, R_2=OH, R_3=OH
4: R_1=OAc, R_2=OH, R_3=OAc
5: R_1=OH, R_2=OH, R_3=H
7: R_1=$OCO(CH_2)_2CH_3$, R_2=OMe, R_3=H

2. Results and Discussion

Krempfielin J (**1**) showed the pseudomolecular ion peak $[M + Na]^+$ at *m*/*z* 417.2616 in the HRESIMS and the molecular formula was determined as $C_{23}H_{38}O_5$. NMR spectroscopic data of **1** (Table 1) showed the presence of one acetoxy group (δ_C 169.6 and 22.4; δ_H 2.12, s, 3H) and one methoxyl group (δ_C 57.0 and δ_H 3.34, 3H, s). The NMR data of **1** was found to be similar to the known compound, **7** [22,25] (Chart 1). Comparison of the NMR data of them revealed that the only difference between both compounds arises from the replacement of the *n*-butyryloxy group at C-3 in **7** by one acetoxy group in **1**. The stereochemistry of **1** was confirmed by comparison of the NMR data and NOE correlations of both **7** and **1**.

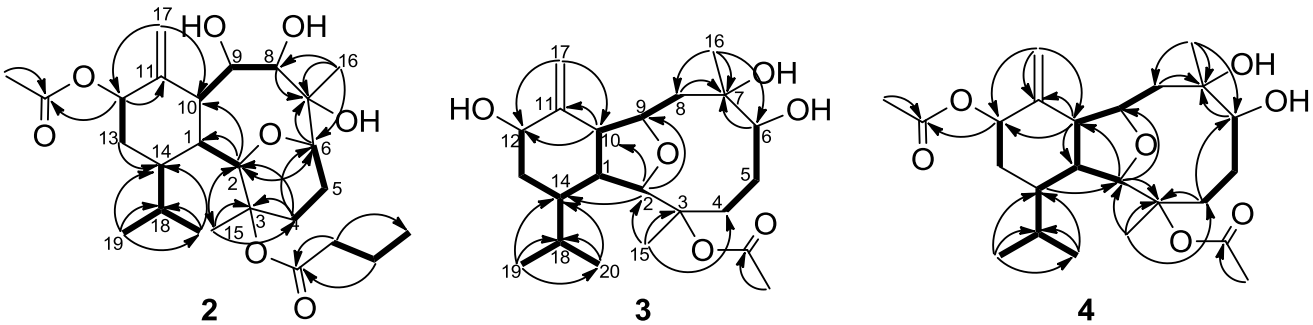
Table 1. ^{13}C and ^{1}H NMR data for compounds **1**–**4**.

	1 [a]		**2** [b]		**3** [a]		**4** [a]	
	δ_C	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**
1	45.6, CH [c]	2.20 dd (10.8, 8.0) [d]	37.8, CH	3.24 m	44.2, CH	2.31 t (8.0)	44.2, CH	2.31 ddd (8.0, 6.0, 2.0)
2	92.1, CH	3.59 br s	80.9, CH	3.58 m	90.4, CH	3.69 br s	90.2, CH	3.69 d (2.0)
3	86.6, C		83.8, C		86.6, C		86.6, C	
4	36.8, CH_2	1.75 m 2.61 dd (14.4, 9.2)	28.8, CH_2	1.92 m 2.90 dd (14.8, 7.0)	34.6, CH_2	1.94 m 2.48 m	34.9, CH_2	1.92 m 2.52 dd (14.8, 8.8)
5	26.8, CH_2	1.33 m 1.59 m	19.9, CH_2	1.50 m 1.71 m	30.4, CH_2	1.42 m 1.71 m	30.5, CH_2	1.45 m 1.68 m
6	90.6, CH	4.12 d (6.0)	70.1, CH	3.68 m	79.0, CH	4.57 d (8.4)	79.1, CH	4.58 d (7.2)
7	75.9, C		75.5, C		76.6, C		76.5, C	
8	45.0, CH_2	1.82 m	79.0, CH	3.62 m	46.0, CH_2	1.85 m	45.9, CH_2	1.83 m
9	78.5, CH	4.18 dd (14.8, 7.2)	69.9, CH	4.39 t (8.0)	79.4, CH	4.53 m	78.8, CH	4.40 m
10	53.9, CH	2.95 t (7.2)	50.0, CH	2.51 br s	51.0, CH	2.86 m	51.4, CH	2.93 dd (7.2, 6.0)
11	147.6, C		145.3, C		148.0, C		143.0, C	
12	31.5, CH_2	2.04 m 2.25 m	71.6, CH	5.29 m	70.7, CH	4.37 s	72.5, CH	5.44 dd (5.6, 2.8)
13	24.6, CH_2	1.00 m 1.70 m	31.9, CH_2	1.58 m 2.17 m	31.2, CH_2	1.46 m 1.84 m	29.0, CH_2	1.42 m 1.90 m
14	44.0, CH	1.26 m	40.1, CH	1.48 m	36.5, CH	1.82 m	37.2, CH	1.70 m
15	23.1, CH_3	1.41 s	26.3, CH_3	1.56 s	23.2, CH_3	1.48 s	23.2, CH_3	1.47 s
16	23.6, CH_3	1.13 s	13.7, CH_3	1.19 s	22.6, CH_3	1.17 s	22.8, CH_3	1.18 s
17	109.4, CH_2	4.64 s 4.68 s	105.0, CH_2 [e]	5.04 s 5.23 s	111.8, CH_2	4.87 s 5.06 s	114.9, CH_2	4.97 s 5.14 s
18	29.1, CH	1.72 m	28.3, CH	2.03 m	29.0, CH	1.80 m	28.9, CH	1.80 m
19	15.6, CH_3	0.79 d (6.8)	21.5, CH_3	1.06 d (6.5)	16.4, CH_3	0.85 d (6.4)	16.2, CH_3	0.83 d (6.8)
20	21.9, CH_3	0.97 d (6.8)	21.5, CH_3	1.09 d (6.5)	21.9, CH_3	1.00 d (6.4)	21.8, CH_3	0.96 d (6.8)
3-*n*-butyrate			172.6, C 37.5, CH_2 18.6, CH_2 13.8, CH_3	2.22 m 2.30 m 1.64 m 0.95 t (7.5)				
3-OAc	169.6, C 22.4, CH_3	2.12 s			169.5, C 22.4, CH_3	2.07 s	169.4, C 22.4, CH_3	2.07 s
6-OMe	57.0, CH_3	3.34 s						
6-OAc								
8-OAc								
12-OAc			170.0, C 21.2, CH_3	2.14 s			170.3, C 21.5, CH_3	2.06 s

[a] ^{13}C and ^{1}H spectra recorded at 100 and 400 MHz in $CDCl_3$; [b] ^{13}C and ^{1}H spectra recorded at 125 and 500 MHz in $CDCl_3$; [c] Deduced from DEPT; [d] *J* values (Hz) in parentheses; [e] Broad signal.

The new metabolite krempfielin K (**2**) was found to have the molecular formula $C_{26}H_{42}O_8$ and six degrees of unsaturation, as indicated from the HRESIMS. The IR absorptions at ν_{max} 3444 and 1732 cm^{-1} revealed the presence of hydroxy and ester carbonyl functionalities. The ^{13}C carbons spectral data (Table 1) were assigned by the assistance of DEPT spectrum to six methyls (including one acetate methyl δ_C 21.2), five sp^3 methylenes, one sp^2 methylene, nine sp^3 methines (including five oxymethines), two sp^3 and three sp^2 quaternary carbons (including two ester carbonyls). The NMR spectroscopic data of **2** (Table 1) showed the presence of one 1,1-disubstituted double bond (δ_C 105.0, CH_2 and 145.3, C; δ_H 5.23, s and 5.04, s, each 1H). Two ester carbonyls (δ_C 172.6 and 170.0) were assigned from the ^{13}C NMR spectrum and were HMBC correlated with the methylene (δ_H 2.30 m and 2.22 m, each 1H) of an *n*-butyrate and those of an acetate methyl (δ_H 2.14, s, 3H), respectively. Therefore, the remaining three degrees of unsaturation identified compound **2** as a tricyclic molecule. ^1H-^1H COSY and HMBC correlations (Figure 1) were further used to establish the molecular skeleton of **2**. The COSY experiment assigned five isolated consecutive proton spin systems. Above evidences and HMBC correlations from H-2 and H-6 (δ_H 3.58 and 3.68) to C-6 and C-2 (δ_C 70.1 and 80.9), respectively, implied the presence of an ether linkage between C-2 and C-6, and suggested that **2** is an 2,6-ether linked eunicellin-based diterpenoid [1,26]. Furthermore, the acetoxy group attaching at C-12 was confirmed from the HMBC correlations from H-12 (δ_H 5.29) and acetate methyl protons (δ_H 2.14) to the carbonyl carbon appearing at 170.0 (C). Thus, the remaining one *n*-butyryloxy group had to be positioned at C-3, an oxygen-bearing quaternary carbon resonating at δ 83.8 ppm. On the basis of above analysis, the planar structure of **2** was established. The relative structure of **2** was elucidated by the analysis of NOESY correlations, as shown in Figure 2. The observation of the NOE interactions between H-1 and H-6, H-8 and H-10; H-10 and both H-8 and H-12, revealed that they are all β-oriented. Also, the correlations between H-2 and H-9, H-14 and H_3-15; H-9 and H_3-16 suggested that of all of H-2, H-9, H-14, H_3-15 and H_3-16 are α-oriented. The relative configuration of **2** was thus established. The highly oxygenated pattern of **2** at C-2, C-3, C-6, C-7, C-8, C-9 and C-12 is rare in known eunicellins.

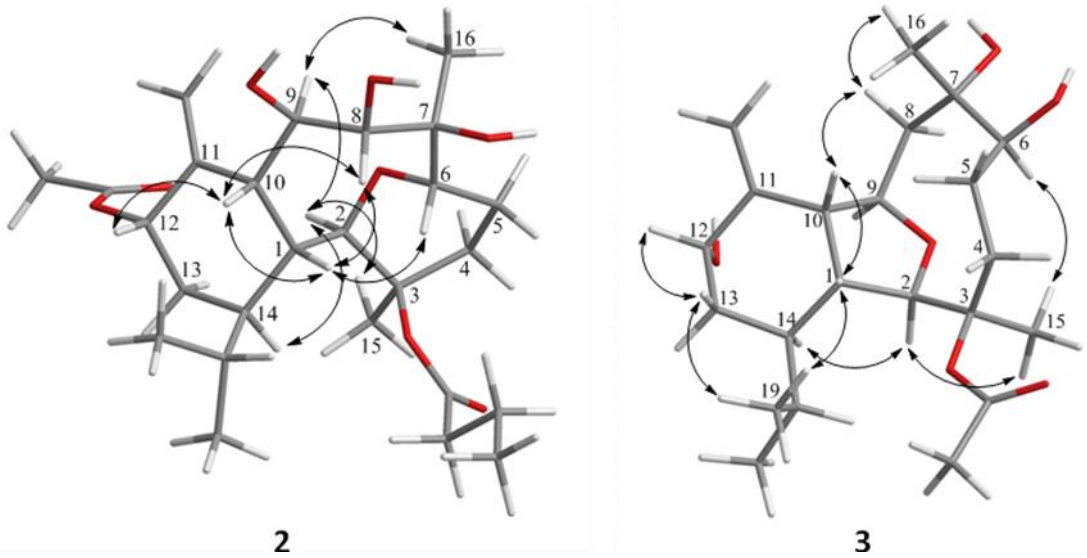
Figure 1. Selected ^1H-^1H COSY (▬) and HMBC (→) correlations of **2**–**4**.



Krempfielin L (**3**) showed the molecular ion peak $[M + Na]^+$ at *m*/*z* 419.2407 in the HRESIMS and established a molecular formula of $C_{22}H_{36}O_6$, implying five degrees of unsaturation. The IR absorptions at ν_{max} 3419 and 1734 cm^{-1} revealed the presence of hydroxy and ester carbonyl functionalities. The ^{13}C NMR spectrum of **3** showed signals of 22 carbons (Table 1), which were characterized by the DEPT spectrum as five methyls (including one acetate methyl), five methylenes (including one sp^2 methylene),

eight methines (including four oxygenated carbons), and four quaternary carbons (including one ester carbonyl and one sp^2 quaternary carbon of an olefin). The ^1H and ^{13}C NMR spectral data of **3** (Table 1) also showed the presence of one acetoxy group (δ_H 2.07, s, 3H; δ_C 22.4, CH_3 and 169.5, C). The remaining three degrees of unsaturation again identified **3** as a tricyclic diterpenoid. The molecular framework was established by ^1H-^1H COSY and HMBC correlations (Figure 1). Comparison of the NMR data of **3** with those of the known compound sclerophytin E [27] revealed that **3** is the C-12 hydroxylated derivative of sclerophytin E. The stereochemistry of compound **3** was determined by the NOESY spectrum as shown in Figure 2.

Figure 2. Key NOESY correlations for **2** and **3**.



The HRESIMS (*m*/*z* 461.2518 $[M + Na]^+$) of krempfielin M (**4**) established the molecular formula of $C_{24}H_{38}O_7$. The ^1H and ^{13}C NMR spectral data of **4** (Table 1) revealed that the structure of metabolite **4** should be similar to that of **3**, as the ^1H NMR spectral data of **4** are almost identical with those of **3** except for the presence of one additional acetyl group (δ_H 2.06, s, 3H) in **4**. Furthermore, the placement of an acetoxy group at C-12 was established by the HMBC experiment which showed correlations from an oxymethine (δ_H 5.44, H-12) and acetate methyl (δ_H 2.06) to the ester carbonyl carbon appearing at δ_C 170.3 (C) (Figure 1). The NOE correlations of **4** also showed that the stereochemistry of this metabolite is identical with that of **3**. Thus the structure of metabolite **4** was determined.

The *in vitro* anti-inflammatory effects of the diterpenoids **1**–**6** were tested by examining the inhibitory activity of these compounds toward the release of elastase in *N*-formyl-methionyl-leucyl-pheny lalanine/cytochalasin B (FMLP/CB)-induced human neutrophils cells (Table 2). At a concentration of 10 μM, all of these compounds could not significantly reduce the expression of superoxide anion, relative to the control cells stimulated with FMLP/CB only. At the same concentration, compounds **2** and **4** were found to effectively inhibit the elastase release (45.51 ± 2.69% and 27.30 ± 5.42% inhibition, respectively) in the same FMLP/CB-stimulated cells.

Table 2. Effect of pure compounds on elastase release in FMLP/CB-induced human neutrophils.

Compound	Elastase		
	Inh%		IC_{50} (μM)
1	19.42 ± 7.89	*	>10
2	45.51 ± 2.69	***	>10
3	18.67 ± 5.75	*	>10
4	27.30 ± 5.42	**	>10
5	−0.32 ± 0.81		>10
6	6.15 ± 3.42		>10

Percentage of inhibition (Inh%) was measured at 10 μM. Results are presented as mean ± S.E.M. (n = 3 or 4); * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control value.

3. Experimental Section

3.1. General Experimental Procedures

Melting point was determined using a Fisher-Johns melting point apparatus. Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESIMS were obtained with a Bruker APEX II mass spectrometer. The NMR spectra were recorded either on a Varian UNITY INOVA-500 FT-NMR and a Varian 400MR FT-NMR. Silica gel (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical thin layer chromatography (TLC). High performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with an octadecylsilane (ODS) column (250 × 21.2 mm, 5 μm).

3.2. Animal Material

C. krempfi was collected by hand using scuba off the coast of Penghu islands of Taiwan in June 2008, at a depth of 5–10 m, and stored in a freezer until extraction. A voucher sample (specimen No. 200806CK) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. Extraction and Separation

The octocoral (1.1 kg fresh wt) was collected and freeze-dried. The freeze-dried material was minced and extracted exhaustively with EtOH (3 × 10 L). The EtOH extract of the frozen organism was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2-soluble portion (14.4 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0%–100% of EtOAc, stepwise) and then further with MeOH in EtOAc with increasing polarity to yield 41 fractions. Fraction 28, eluted with *n*-hexane–EtOAc (1:2), was rechromatoraphed over a reversed-phase column (RP-18) using acetone–H_2O (10:1) as the mobile phase to afford six subfractions (A1–A6). Subfraction A3 was repeatedly separated by reverse phase HPLC (CH_3CN–H_2O, 1.4:1.1) to afford compound **1** (2.6 mg). Fraction 31, eluted with *n*-hexane–EtOAc (1:10), was rechromatoraphed over a silica gel

open column using *n*-hexane–acetone (3:1) as the mobile phase to afford eight subfractions (B1–B8). Subfraction B5 separated by reverse phase HPLC (CH_3CN–H_2O, 1:1 to 1:1.6) to afford compounds **2** (5.1 mg), **3** (2.8 mg), **5** (52.3 mg) and **6** (13.4 mg). Subfraction B6 by reverse phase HPLC (CH_3CN–H_2O, 1:1.5) to afford compounds **4** (8.4 mg).

3.3.1. Krempfielin J (**1**)

Colorless oil; $[\alpha]^{23}_D$ = +52 (*c* 0.85, $CHCl_3$); IR (neat) ν_{max} 3479, 2958, 2930, 2872, 1737, 1644, 1463, 1369, 1244, and 1100 cm^{-1}; ^{13}C and ^1H NMR data, see Table 1; ESIMS *m*/*z* 417 $[M + Na]^+$; HRESIMS *m*/*z* 417.2616 $[M + Na]^+$ (calcd. for $C_{23}H_{38}O_5Na$, 417.2617).

3.3.2. Krempfielin K (**2**)

White powder; mp 162–163 ℃; $[\alpha]^{25}_D$ = −58 (*c* 1.5, $CHCl_3$); IR (neat) ν_{max} 3444, 2963, 1731, 1651, 1464, 1380, 1240, 1177, 1087, and 1045 cm^{-1}; ^{13}C and ^1H NMR data, see Table 1; ESIMS *m*/*z* 505 $[M + Na]^+$; HRESIMS *m*/*z* 505.2780 $[M + Na]^+$ (calcd. for $C_{26}H_{42}O_8Na$, 505.2777).

3.3.3. Krempfielin L (**3**)

Colorless oil; $[\alpha]^{25}_D$ = +26 (*c* 0.8, $CHCl_3$); IR (neat) ν_{max} 3419, 2958, 1734, 1457, 1369, 1246, 1063 and 1026 cm^{-1}; ^{13}C and ^1H NMR data, see Table 1; ESIMS *m*/*z* 419 $[M + Na]^+$; HRESIMS *m*/*z* 419.2407 $[M + Na]^+$ (calcd. for $C_{22}H_{36}O_6Na$, 419.2409).

3.3.4. Krempfielin M (**4**)

Colorless oil; $[\alpha]^{25}_D$ = +24 (*c* 2.4, $CHCl_3$); IR (neat) ν_{max} 3452, 2960, 1736, 1435, 1370, 1243, 1199, 1075, and 1024 cm^{-1}; ^{13}C and ^1H NMR data, see Table 1; ESIMS *m*/*z* 461 $[M + Na]^+$; HRESIMS *m*/*z* 461.2518 $[M + Na]^+$ (calcd. for $C_{24}H_{38}O_7Na$, 461.2515).

3.4. In Vitro *Anti-Inflammatory Assay—Superoxide Anion Generation and Elastase Release by Human Neutrophils*

Human neutrophils were obtained by means of dextran sedimentation and Ficoll centrifugation. Measurements of superoxide anion generation and elastase release were carried out according to previously described procedures [28,29]. LY294002, a phosphatidylinositol-3-kinase inhibitor, was used as a positive control for inhibition of superoxide anion generation and elastase release with IC_{50} values of 1.88 ± 0.45 and 4.12 ± 0.92 μM, respectively. Briefly, superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome c. Elastase release experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate [30].

4. Conclusions

New eunicellin-based diterpenoids were isolated together with known ones from the soft coral *Cladiella krempfi*. Compounds **2** and **4** could significantly inhibit the release of elastase in

FMLP/CB-induced human neutrophils. Thus, compounds **2** and **4**, in particular **2**, could be promising anti-inflammatory agents and may warrant further biomedical investigation.

Acknowledgements

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